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Studies on effect of alkali pretreatment of banana pseudostem for fermentable sugar production for biobutanol production

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Abstract

Aim : In this study, effectiveness of alkali pretreatment of banana pseudostem for the production of biobutanol by fermentation using *Clostridium sporogenes* under strict anaerobic conditions was studied.

Methodology : Banana pseudostem was pretreated by alkali soaking at constant temperature (30, 50 and 70°C) and concentration of 0.25 to 2% (w/v) at solid to liquid ratio of 1:10 for 24 hr to depolymerize the lignocellulosic biomass. Further to increase delignification, the alkali soaked samples were subjected to thermal treatment at 121°C for 15 min. The hydrolysates were subjected to acetone-butanol-ethanol fermentation using *Clostridium sporogenes* to produce butanol.

Results : Maximum delignification of 92% was obtained at 30°C soaking temperature with 2% NaOH (w/v). Increase in temperature led to increase in delignification but also resulted in reduced recovery of cellulose. Maximum glucose yield of 524 mg g⁻¹ glucan was obtained from the pretreated biomass. Fermentation of hydrolysate obtained from alkali soaking pretreatment by *C. sporogenes* under anaerobic condition resulted in 10.12 g l⁻¹ of butanol with a yield of 0.326 g g⁻¹ of total sugar consumed and it was much higher compared to untreated biomass.

Interpretation : Alkali soaking pretreatment at 30°C and alkali concentration of 1.5% (w/v) was effective to depolymerize the biomass to obtain maximum sugar and butanol yield from banana pseudostem. Thermal treatment was not effective in increasing the production of butanol due to pseudo lignin formation and presence of inhibitors.

Key words: Alkali pretreatment, Banana pseudostem, Biobutanol, *Clostridium sporogenes*, Enzymatic hydrolysis

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Introduction

Biofuels are expected to play a major role in ensuring future energy security by reducing the fossil fuel dependency and mitigating climate change due to emission of greenhouse gases. Biobutanol is a next generation biofuel and considered superior to bioethanol due to higher energy density, lower Reid vapour pressure, easy blending with gasoline at any ratio and ease in transportation with the existing infrastructure (Bellido *et al.*, 2014; Qureshi and Ezeji, 2008). It can be produced from renewable lignocellulosic wastes generated by agriculture and forestry (Diaz *et al.*, 2013; Sellin *et al.*, 2013). Banana pseudostem is a potential lignocellulosic biomass and has been evaluated for bioethanol production (Gabhane *et al.*, 2014; Ingale *et al.*, 2014). It has been reported as one of the most under-utilized biomass with great potential (Farooq *et al.*, 2012; Padam *et al.*, 2014). India is the largest producer of banana in the world and for each ton of banana harvest around three tons of banana pseudostem is generated (Souza *et al.*, 2014). Lignocellulosic biomass mainly consists of cellulose, hemicellulose and lignin. In order to produce biofuel, lignocellulosic biomass based biorefinery involves four main steps namely, pretreatment, hydrolysis, fermentation and product separation. Pretreatment is a critical step and has significant impact on other steps of biorefinery process (Galbe and Zacchi, 2012). Due to complexity of lignocellulosic biomass matrix, usually a high energy input is required to maximize the sugar release during pretreatment (Yamakawa *et al.*, 2018).

Pretreatment of lignocellulosic biomass is necessary to depolymerise the biomass by altering / removing lignin and to improve the accessibility of enzyme to cellulose for hydrolysis (Kumar *et al.*, 2009). The methods employed in pretreatment include physical, physiochemical, chemical and biological techniques (Kumar and Sharma, 2017). Depolymerisation using alkaline treatment is one of the leading pretreatment methods for improving cellulose digestibility of lignocellulosic materials to degrade lignin from biomass and to recover cellulosic material (Balakrishnaraja *et al.*, 2017; Mosier *et al.*, 2005). In light of the above, the present study was carried out to investigate the effectiveness of alkali pretreatment of banana pseudostem for the production of biobutanol by anaerobic fermentation using *Clostridium sporogenes*.

Materials and Methods

Plant material: Samples of banana pseudostem were purchased from market in Chennai, (India), cut into 2 cm size pieces and washed to remove sand and dirt. It was dried in sunlight followed by heating in oven at 60°C till constant weight was obtained. The dried samples were ground to <25 mesh size and stored in air tight container for further use. Lyophilized *Clostridium sporogenes* (NCIM 2337) was procured from National Chemical Laboratory, Pune, India.

Pretreatment: In this study, two different pretreatments were carried out: Soaking banana pseudostem in NaOH solution (0.25-2.0% w/v) for 24 hr at 30-70°C under shaking condition at a solid

to liquid ratio of 1:10, followed by thermal treatment at 121°C and 15 psi for 15 min. After the treatment, solids were recovered by filtration, washed with water till neutral pH was observed and then dried at 60°C till constant weight was obtained. All the experiments were carried out in triplicate and the average values were reported and standard deviations were represented as error bars.

Enzymatic hydrolysis: Enzymatic hydrolysis of untreated and pretreated banana pseudostem was carried out at a solid loading of 1:10 in 50 mM citrate buffer (pH 4.8) containing 50 FPU g⁻¹ biomass of cellulase and 350 U g⁻¹ biomass of β-glucosidase. In addition, sodium azide (0.02%) was added to prevent fungal growth. The contents were incubated in an orbital shaker at 50°C and 120 rpm for 24 hr (Selig *et al.*, 2008) and at the end of the reaction, contents were centrifuged at 8000 rpm for 15 min. All the experiments were carried out in triplicate and the average values were reported and standard deviations were represented as error bars.

Anaerobic culture maintenance and fermentation: *C. sporogenes* spores were revived anaerobically inside an anaerobic culture jar system (HiMedia) in Reinforced Clostridia Agar and Reinforced Clostridia Medium at 37°C for 24 hr. Culture was maintained as spore suspension in sterile water at 4°C. The inoculum was prepared in Clostridia medium containing 5 g l⁻¹ glucose, 5 g l⁻¹ yeast extract, 3 g l⁻¹ starch, 10 g l⁻¹ beef extract, 10 g l⁻¹ peptone, 5 g l⁻¹ sodium chloride, 3 g l⁻¹ sodium acetate, 0.5 g l⁻¹ agar and 0.5 g l⁻¹ cysteine hydrochloride. The pH of the medium was 7. About 100 ml of this medium was sterilized at 121°C and 15 psi, cooled to room temperature and inoculated with *C. sporogenes* culture. This was incubated for 24 hr at 37 ± 0.5°C inside an anaerobic culture jar until active growth was observed.

Fermentation was carried out in 150 ml Erlenmeyer flasks in anaerobic culture jar using enzymatic hydrolysate as substrate. Hydrolysates containing higher sugar concentration from both the pretreatment methods were subjected to fermentation. Nutrients were added to the hydrolysate sample and was sterilized in an autoclave, cooled and inoculated with 10% (v/v) actively growing *Clostridium sporogenes* culture. Samples were collected at regular intervals (24 hr) to monitor the growth pattern and acetone-butanol-ethanol production. The yield is the ratio of amount of product obtained and total sugars utilized and productivity is the ratio of solvent production (g l⁻¹) and fermentation time and was calculated as described by Qureshi *et al.* (2010).

Analytical methods: Cellulose, hemicellulose and lignin contents present in banana pseudostem were estimated by detergent extraction method (Goering and Van Soest, 1970). Fourier transform infrared spectroscopy (FTIR) with a detector at 4 cm⁻¹ resolution and 25 scan per sample was used to analyse the changes in functional groups. Scanning electron microscopy (SEM) was used to investigate the morphology of pretreated sample. The crystalline nature of banana pseudostem before and

after pretreatment was analysed using GE X-ray diffraction system. The samples were scanned in the range of $2\theta = 5\text{--}35^\circ$ with a step size of 0.02° and the crystallinity index (CrI) was determined by Eq. (1) (Segal *et al.*, 1959).

$$\%CrI = \left\{ \frac{I_{002} - I_{am}}{I_{002}} \right\} \times 100 \quad (1)$$

where, I_{002} is the intensity of diffraction (002) plane at about $2\theta = 22.5^\circ$ and I_{am} is the intensity of baseline at about $2\theta = 18.4^\circ$. The solid recovery after pretreatment was calculated by Eq. 2 (Gu *et al.*, 2013).

$$\% \text{ Solid recovery} = \left\{ \frac{\text{Dry pretreated solid (g)}}{\text{Dry raw material (g)}} \right\} \times 100 \quad (2)$$

The % delignification and % cellulose recovery were calculated by Eq.3 and 4 (Haykir *et al.*, 2013):

$$\% \text{ Delignification} = \left\{ \frac{L_{unS} - L_{PTS}}{L_{unS}} \right\} \times 100 \quad (3)$$

$$\% \text{ Cellulose recovery} = \left(\frac{C_{PTS}}{C_{unS}} \right) \times 100 \quad (4)$$

where, L_{unS} is the lignin content in untreated sample and L_{PTS} is the lignin content in pre-treated sample expressed in g g^{-1} , C_{PTS} is the cellulose content of pre-treated sample and C_{unS} is the cellulose content of untreated sample expressed in g g^{-1} . Monosaccharide sugars were analysed by high performance liquid chromatography (HPLC) equipped with refractive index detector (Agilent 1260 – Hi-Plex H column), using 5 mM sulphuric acid as mobile phase at a flow rate of 0.6 ml min^{-1} at 65°C oven

temperature and the fermentation products were analysed by HPLC with refractive index detector using HPLC grade water as mobile phase at a rate of 0.6 ml min^{-1} at 40°C oven temperature as recommended by the manufacturer and the analytical methods described in the literature (Kumar *et al.*, 2014; Tsuey *et al.*, 2006).

Results and Discussion

The amount of cellulose, hemicellulose and lignin present in untreated sample were 41.04, 17.39 and 10.51%, respectively. This is in agreement with the results reported in literature Jayaprabha *et al.* (2011). Increase in alkali concentration and soaking temperature resulted in decreased solid recovery. As the concentration of alkali increased, cellulose recovery increased in alkali soaking method (Fig. 1). When the sample was further subjected to thermal treatment, cellulose recovery was reduced indicating higher dissolution of cellulose at higher alkali concentration. Cellulose recovery varied between 62.94 and 74.01% in alkali soaking pretreatment. With additional thermal pretreatment, the cellulose recovery varied between 53.13 and 86.27%. Similar results were reported for low concentration alkali pretreatment of corncob (Sharma *et al.*, 2017). It was also observed that the lignin was removed using lesser alkali concentration (up to 1% NaOH) and got precipitated during thermal treatment (Fig. 2). In case of higher alkali concentration, additional thermal treatment was effective in delignification. Maximum delignification of 91.27 and 91.52% was obtained at 30°C soaking temperature with 1.5 and 2.0% (w/v) NaOH, respectively, with additional thermal treatment. Under similar conditions, in the absence of thermal pretreatment, 57.33 and 77.57% delignification was observed.

Fig. 1: Effect of NaOH concentration and soaking temperature on % cellulose recovery (A) Alkali soaking pretreatment and (B) Alkali soaking + Thermal pretreatment.

Fig. 2: Effect of NaOH concentration and soaking temperature on %delignification (A) Alkali soaking pretreatment and (B) Alkali soaking + Thermal pretreatment.

Fig. 3: SEM images of (A) untreated banana pseudostem, (B) pretreated banana pseudostem (conditions: 1:10 solid liquid ratio, 1.5% (w/v) NaOH soaking at 30°C for 24 hr), (C) hydrolysis residue (conditions: 5 g pretreated biomass, 50 ml mM citrate buffer, 50°C, pH 4.8, cellulase 50 FPU g⁻¹ biomass and β-glucosidase 350 U g⁻¹ biomass).

Fig. 4: XRD pattern of banana pseudostem: untreated, pretreated and hydrolysis residue (pretreatment condition: 1:10 solid liquid ratio, 1.5% (w/v) NaOH soaking at 30°C for 24 hr and hydrolysis condition: 5 g pretreated biomass, 50 ml mM citrate buffer, 50°C, pH 4.8, cellulase 50 FPU g⁻¹ biomass and β-glucosidase 350 U g⁻¹ biomass).

Fig. 5: FTIR spectra of banana pseudostem: (A) untreated, (B) pretreated and (C) hydrolysis residue (pretreatment condition: 1:10 solid liquid ratio, 1.5% (w/v) NaOH soaking at 30°C for 24 hr and hydrolysis condition: 5 g pretreated biomass, 50 ml mM citrate buffer, 50°C, pH 4.8, cellulase 50 FPU g⁻¹ biomass and β-glucosidase 350 U g⁻¹ biomass).

Hence, it can be concluded that thermal pretreatment had significantly improved delignification.

It was observed that the pretreatment had induced significant physical changes in the biomass. Smooth and continuous surface was observed from the SEM images of untreated sample (Fig. 3A). Smaller porosities and undamaged fibrous organization were found in the smoother walls of untreated banana pseudostem sample (Bilba *et al.*, 2007; Shimizu *et al.*, 2018). Damaged and relatively less smooth surface with pores were observed in thermally treated sample. SEM image of hydrolysis residue exhibited pores of higher sizes and completely damaged cell wall, confirming the release of sugars (Fig. 3C).

X-ray diffraction analysis of untreated, pretreated and hydrolysed banana pseudostem was performed and is shown in Fig. 4. Crystallinity index (Crl) is an indication of the extent of material destruction. Crl of untreated sample was found to be 34% and it was closer to the value of 39% reported earlier by Guimarães *et al.* (2009). The Crl of pretreated sample increased to 57%, indicating partial hydrolysis of hemicellulose and removal of lignin caused by alkali pretreatment. On further enzymatic hydrolysis, the amorphous cellulose was easily digested by enzyme mixture leaving behind the crystalline cellulose resulting in 55% Crl. There was no direct relationship between digestibility and Crl value, but there was a close relationship with hemicellulose and lignin removal (Yu *et al.*, 2013a).

Fourier Transform Infrared Spectroscopy was used to investigate the changes in cellulose structures during alkali

pretreatment and is shown in Fig. 5. The prominent bands observed at 1000 - 1200 cm⁻¹ indicated the structural features of cellulose and hemicellulose, and the enhanced absorption peaks in this region indicated the elevated levels of cellulose, was similar to the results reported by Zhao *et al.* (2010). The peak at 1160 cm⁻¹ indicated modification in cellulose. The absorption at 2910 cm⁻¹ represents the stretching of -CH₃ and -CH₂ indicating the changes due to pretreatment (Binod *et al.*, 2009).

Enzymatic hydrolysis was used to convert cellulose and hemicellulose into hexose (glucose) and pentose sugars (xylose and arabinose). The pretreated and untreated banana pseudostem samples were hydrolysed using cellulase (50 FPU g⁻¹ biomass) supplemented with β-glucosidase (350 U g⁻¹ biomass) in order to obtain maximum glucose yield. Fig. 6 shows the effect of NaOH concentration and soaking temperature on glucose yield from pretreated sample by alkali soaking (BPSASEH) and alkali soaking, followed by thermal treatment (BPSASTEH) and untreated sample (BPSEH). Glucose yield decreased with an increase in soaking temperature. Maximum glucose yield of 524 mg g⁻¹ glucan was obtained from the sample treated at 30°C with 1.5% NaOH. In case of sample treated under similar conditions with additional thermal treatment resulted in lesser yield of 431 mg g⁻¹ glucan, inspite of higher delignification (91.27%), this might be due to the condensation of lignin pseudo molecule hindering hydrolysis as reported by Yu *et al.* (2013b). Distribution of lignin and its modification is important for the enzymatic digestibility of lignocellulosic biomass rather than its removal (Taherzadeh and Karimi, 2008; Yang and Wyman, 2004). In this study, alkali pretreatment (30°C with

Fig. 6: Effect of NaOH concentration of glucose yield from banana pseudostem (conditions: 5g untreated / pretreated biomass, 50ml mM citrate buffer, 50°C, pH 4.8, cellulase 50 FPU g⁻¹ biomass and β -glucosidase 350 U g⁻¹ biomass) (A) Soaking pretreatment and (B) Soaking + Thermal pretreatment.

Fig. 7: Comparative analysis of fermentation products by fermentation of BPSEH, BPSASEH, BPSASTEH using *Clostridium sporogenes* at 37°C, pH 7 and 10% inoculum.

1.5% NaOH) improved the glucose yield by 2.53 times compared to untreated banana pseudostem.

Samples with higher sugar concentration obtained from hydrolysis process were subjected to fermentation using *C. sporogenes* under strict anaerobic conditions. Fermentation profile of product formation and sugar was studied for seven days. The concentration of ethanol and butanol were found to be constant after 72 hr of fermentation and the concentration of acetone was further found to be increased. Hence, all the results

were compared at the end of 72 hr of fermentation and the results are presented in Fig. 7. Initial glucose concentration of fermentation medium after the addition of nutrients and inoculum were 12.26, 29.23 and 24.9 g l⁻¹ for untreated banana pseudostem (BPSEH), sample pretreated by alkali soaking (BPSASEH) and alkali soaking followed by thermal pretreatment (BPSASTEH), respectively. Xylose present in BPSEH, BPSASEH and BPSASTEH were 5.16, 3.12 and 2.72 g l⁻¹. Anaerobic fermentation of BPSEH using *C. sporogenes* resulted in 5.87, 4.24 and 2.42 g l⁻¹ of acetone, butanol and ethanol, respectively, with butanol yield of 0.276 g g⁻¹ and total sugar consumption was 15.36 g l⁻¹ including 3.1 g l⁻¹ of xylose. Fermentation of BPSASEH produced 13.37, 10.12 and 4.72 g l⁻¹ of acetone, butanol and ethanol, respectively, with butanol yield of 0.326 g g⁻¹ by consuming 31.04 g l⁻¹ of total sugar including 1.81 g l⁻¹ xylose. However, fermentation of BPSASTEH produced 13.29, 7.57 and 3.1 g l⁻¹ of acetone, butanol and ethanol, respectively, with butanol yield of 0.288 g g⁻¹ by consuming 26.26 g l⁻¹ of total sugar of which 1.36 g l⁻¹ was xylose. The lesser yield of butanol from hydrolysate indicated the presence of inhibitors during pretreatment.

Hence, it can be concluded that the structural modification of lignin is sufficient to maximise the sugar and butanol yield than its removal. The evaluated pretreatment method for banana pseudostem requires less energy and is suitable for tropical countries like India. Banana pseudostem can be a potential feedstock for the production of biobutanol.

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